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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Bacterial pheromones and uses Therefor

5. Name of your agent *(if you have one)*

Fry Heath & Spence

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Dr V A Price - 01293 776880

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BACTERIAL PHEROMONES AND USES THEREFOR

Field of the invention

The present invention relates to RP-factors, their cognate receptors, convertases, respective genes and to inhibitors or mimetics thereof. In particular, the invention relates to antibodies, pharmaceutical compositions and (therapeutic, diagnostic) methods based on the RP-factors and their receptors/convertases.

Introduction

Bacterial pheromones

It is known that certain chemicals may mediate intercellular communication in bacterial cultures. Such communication has been shown to be of importance during sporulation, conjugation, changes in virulence and in bioluminescence. It is now clear that a variety of different autocrine chemical compounds ("pheromones") produced as secondary metabolites are responsible for such social behaviour in prokaryotes (see e.g. Kell et al, 1995, Trends Ecol. Evolution, 10, 126-129).

Pheromones may be distinguished from nutrients *inter alia* in that: (i) they are produced by the organisms themselves, (ii) they are active at very low concentrations (e.g. at picomolar or nanomolar concentrations), and (iii) with the exception of prohormone processing, their *metabolism* is not necessary for activity (although they may of course ultimately be degraded).

The chemical nature of these pheromonal compounds varies widely: those associated with Gram-negative organisms tend to be of low molecular weight (e.g. N-acyl homoserine lactone derivatives), whilst a number of Gram-positive organisms use proteins and polypeptides (Kell et al,1995, *ibidem*).

Pheromones are also known to play an important role in the development of bacterial cultures. For unstressed (uninjured) bacteria and optimal growth media, the "self-promoting" mode of culture growth is normally masked due to the high rate of production of growth factors and the sensitivity of the cells to these pheromones. Only under unfavourable conditions (for example, poor growth media, small initial inocula and/or starved cells) is this behaviour "visible".

For example, a dramatic reduction in the length of the lag phase of cultures of *Nitrosomonas europea* is mediated by N-(3-oxo-hexanoyl) homoserine lactone, and chorionic gonadotropin-like ligand (a 48kD protein) had similar growth-stimulating activity for *Xanthomonas maltophila*. A number of mammalian hormones (including peptide and steroid hormones as well as cytokines) have also been shown to exhibit potent growth-stimulating activities for both Gram-positive and Gram-negative bacteria.

Latency and resuscitation

The ability of a microbial cell to grow and divide on a nutrient agar plate constitutes the benchmark method for determining the number of living cells in a sample of interest. However, it is also widely recognised that, especially in nature, the distinction between life and non-life is not absolute; many cells may exist in "dormant" or "moribund" forms or states and will not produce colonies on nutrient media (i.e. are "non-culturable"). However, these dormant or latent cells are not dead: they can be returned, by a process known as resuscitation, to a state of viability/ culturability.

For example, it is known that cells of the (high-G+C Gram-positive) bacterium *Micrococcus luteus* can enter a state of true dormancy from which they may be resuscitated by culture supernatants, even in the absence of any 'initially viable' cells.

The latent state has profound medical implications: many pathogenic bacteria (including pathogenic mycobacteria such as M. tuberculosis) are known to persist for extended periods in latent states in a host organism. Indeed, tuberculosis is a re-emergent infection of great concern, and it is recognised in particular that the causative organism (*Mycobacterium tuberculosis*) can lie dormant (remain latent) in patients and carriers for periods of years.

The latent state also has important commercial implications, since it complicates many laboratory methods for the detection, cultivation and enumeration of bacteria (for example in the food and healthcare industries).

There is therefore a pressing need to understand the physiological bases of latency and resuscitation.

Summary of the invention

The present invention is based, at least in part, on the discovery of a new class of pheromones which stimulate the resuscitation of bacteria after true dormancy. This 'resuscitation factor' (herein referred to as "RP-factor") may exhibit activity at picomolar concentrations (implying a non-nutritional role).

Thus, in a first aspect of the present invention there is provided an isolated RP-factor.

The term "isolated" is used herein to indicate that the factor exists in a physical milieu distinct from that in which it occurs in nature. For example, the isolated factor may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. The absolute level of purity is not critical, and those skilled in the art can readily determine appropriate levels of purity according to the use to which the factor is to be put.

In many circumstances, the isolated factor will form part of a composition (for example a more or less crude extract containing many other proteins and substances), buffer system or pharmaceutical excipient, which may for example contain other components (including other proteins, such as albumin).

In other circumstances, the isolated protein may be purified to essential homogeneity, for example as determined by PAGE or column chromatography (for example HPLC). In preferred embodiments, the isolated RP-factor of the invention is essentially the sole active RP-factor in a given composition. Particularly preferred are compositions in which an RP-factor (or a particular species, homologue, mutein, derivative or equivalent thereof) is present as the sole active ingredient in a pharmaceutical composition.

The RP-factor for use in the invention need not be isolated in the sense defined above, however. For example, more or less crude culture supernatants (e.g. "spent" medium) may contain sufficient concentrations of RP-factor for use in several applications. Preferably, such supernatants are fractionated and/or extracted (see below), but in many circumstances they may be used without pretreatment. They are preferably derived from spent media used to culture RP-factor-producing microorganisms (for example, the bacterial sources described *infra*). The supernatants are preferably sterile. They may be treated in various ways, for example by concentration, filtration, centrifugation, spray drying, dialysis and/or lyophilisation. Conveniently, the culture supernatants are simply centrifuged to remove cells/cell debris and filtered.

Such supernatants find utility in diagnostic kits and methods, for example in the diagnostic kits and methods described *infra*. They also find utility in the recovery from various samples of culturable microorganisms (e.g. from soil, food, marine, freshwater, or tissue samples, or from samples taken from an organism (e.g. a human or animal).

The culture supernatants may also be used as supplements in various culturing substrates, for example in culture or transport media. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example Mycobacterium spp..

The term "isolated" as applied to the other materials of the invention (for example, the genes and other nucleic acids encoding the RP-factor and their cognate receptors/convertases) is to be interpreted *mutatis mutandis*. Thus, as applied to nucleic acid (e.g. RNA or DNA or (structural) genes), the isolated nucleic acid may be present in any of a wide variety of vectors and in any of a wide variety of host cells (or other milieu, such as buffers, viruses or cellular extracts).

The term "RP-factor" is used herein to encompass any representative of that family of substances the members of which are capable of resuscitating dormant, moribund or latent cells (e.g. dormant bacterial cells). In addition, the RP-factors of the invention may also exhibit growth-stimulatory activity with respect to growing cells (e.g. growing bacterial cells), and/or may be competent to reduce the lag time of cell (e.g. bacterial cell) cultures.

The resuscitation activity (and optionally also the growth-stimulatory activity or lag-time reducing activity) of the RP-factor may be specific for a particular (bacterial) cell (e.g. specific for one or more pathogenic mycobacteria), or may be non-specific. Specificity may be manipulated for example by engineering (e.g. by mutagenesis or chimaerisation, as herein described) of the specificity-determining domain(s) of the RP-factor.

The term "family", as applied to the proteins of the invention, is used herein to indicate a group of proteins which share substantial sequence similarities, either at the level of the primary sequence of the proteins themselves, or at the level of the DNA encoding them. The sequence similarities may extend over the entire protein/gene, or may be limited to particular regions or domains. Similarities may be based on nucleotide/amino acid sequence identity as well as similarity (for example, those skilled in the art recognise certain amino acids as similar, and identify differences based on switches of similar amino acids as conservative changes). Some members of a protein family may be related in

the sense that they share a common evolutionary ancestry, and such related proteins may herein be referred to as homologues. The members of a protein family do not necessarily share the same biochemical properties or biological functions, though their similarities are usually reflected in common functional features (such as effector binding sites and substrates).

The criteria by which protein families are recognised are well-known in the art, and include computer analysis of large collections of sequences at the level of DNA and protein as well as biochemical techniques such as hybridisation analysis and enzymatic assays (see for example Pearson and Lipman (1988), PNAS USA, 85: 2444).

Thus, the RP-factors of the invention include the five factors shown in Fig. 1, together with their species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms (see *infra*). Preferably, the RP-factors of the invention are species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms of any one of the five proteins represented in Fig. 1.

At least two classes of RP-factor may exist: secreted and non-secreted. The secreted RP-factors are characterised by the presence of a secretory signal sequence (the presence of which is readily recognised by those skilled in the art on the basis of the presence of DNA and/or amino acid sequence motifs). The non-secreted RP-factors may be cell-associated or cytosolic factors. Both classes of RP-factor may exist in a single cellular source (e.g. in a single bacterial source). Both classes of RP-factor find application in the invention.

The RP-factors may be synthesised in the form of a precursor which is processed to produce a mature form. Such processing may proceed *via* various intermediate (pro-) forms. Such precursors, intermediate forms and mature proteins are all intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise. As used herein, the term "pro-RP-factor" specifically defines any of various precursors (which may or may not be active) of a mature RP-factor.

The processing may comprise proteolytic cleavage and/or secretion. The precursors may be inactive, and become active on processing as a mature form. The precursors may comprise proteins having secretory leader sequences which are removed during secretion (pre- forms). Such forms are herein referred to as "pre-RP-factor or pre-pro-RP-factors". As explained above, such pre- or prepro- forms are also intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise.

Processing may be attendant on the binding of an RP-factor precursor to a cognate receptor. Such receptors may then directly (or indirectly) cleave the precursor to produce a more mature form of the RP-factor. Such processing may occur as a cascade, involving several receptor-processing complexes, and so ultimately result in the production of a mature RP-factor which then acts as a signalling moiety by binding to a terminal (signal transducing) receptor.

In such processing, the proximal (or intermediate) receptors may function as convertases, and the terminal receptor as a signal transducer. However, a receptor may function as both a convertase and a signal transducer. As used herein, the term "convertase" is intended to define a molecule which binds an RP-factor precursor and (directly or indirectly) processes it to produce a more mature form. They may, for example, have proteases activity.

The receptors/convertases discussed above may be disposed at the cell surface (e.g. membrane bound), cytosolic or extracellular.

Preferably, the RP-factor is derived from a bacterium (e.g. a pathogenic bacterium). Particularly preferred are RP-factors derived from high G + C Gram-positive bacteria.

The term "derived from" as applied to a defined source is intended to define not only a source in the sense of it being the *physical* origin for the material, but also to define material which has structural and/or functional characteristics which correspond to those of material which does originate from the reference source. Thus, a protein "derived from" a given source need not necessarily have been purified from that source.

The term "high G + C Gram-positive bacterium" is a term of art defining a particular class of evolutionarily related bacteria. The class includes Micrococcus spp. (e.g. M. luteus), Mycobacterium spp. (for example a fast- or slow-growing mycobacterium, e.g. M. tuberculosis, M. leprae, M. smegmatis or M. bovis), Streptomyces spp. (e.g. S. rimosus) and Corynebacterium spp. (e.g. C. glutamicum). Preferred according to the invention are RP-factors/cognate receptors/convertases derived from mycobacteria ("mycobacterial RP-factors/RP-factor receptors/convertases").

The invention also contemplates homologues, allelic forms, species variants, derivatives, muteins or equivalents of the RP-factors and RP-factor receptors/convertases of the invention.

The term "homologue" is used herein in two distinct senses. It is used sensu stricto to define the corresponding protein from a different organism (i.e. a *species variant*), in which case there is a direct evolutionary relationship between the protein and its homologue. This may be reflected in a structural and functional equivalence, the protein and its homologue performing the same role in each organism.

The term is also used herein sensu lato to define a protein which is structurally *similar* (i.e. not necessarily related and/or structurally and functionally equivalent) to a given (reference) RP-factor. In this sense, homology is recognised on the basis of purely structural criteria by the presence of amino acid sequence identities and/or conservative amino acid changes (as set out by Dayhoff *et alia*, *Atlas of protein structure* vol. 5, National BioMed Fd'n, Washington D.C., 1979).

For the purposes of the invention, homologues may be recognised as those proteins the corresponding DNAs of which are capable of specifically or selectively cross-hybridising, or which can cross-hybridise under selective, appropriate and/or appropriately stringent hybridisation conditions.

The term "selectively or specifically (cross)hybridisable" in this context indicates that the sequences of the corresponding ssDNAs are such that binding to a unique (or small class) of homologous sequences can be obtained under more or less stringent hybridisation conditions. This method of the invention is not dependent on any particular hybridisation conditions, which can readily be determined by the skilled worker (e.g. by routine trial and error or on the basis of thermodynamic considerations).

Preferably, the homologues, derivatives, muteins or equivalents of the RP-factor of the invention have at least 20% identity with any one of the particular amino acid sequences shown in Fig. 1.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% identity, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with any one of the particular amino acid sequences shown in Fig. 1.

The homologues, derivatives, muteins or equivalents of the RP-factor of the invention may have at least 25% homology with any one of the particular amino acid sequences shown in Fig. 1.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% homology with any one of the particular amino acid sequences shown in Fig. 1.

The term "derivative" as applied herein to the proteins (e.g. the RP-factors or RP-factor receptors/convertases) of the invention is used to define proteins which are modified versions of the proteins of the invention. Such derivatives may include fusion proteins, in which the proteins of the invention have been fused to one or more different proteins or peptides (for example an antibody or a protein domain conferring a biochemical activity, to act as a label, or to facilitate purification).

The derivatives may also be products of synthetic processes which use a protein of the invention as a starting material or reactant.

The term "mutein" is used herein to define proteins that are mutant forms of the proteins of the invention, i.e. proteins in which one or more amino acids have been added, deleted or substituted. The muteins of the invention therefore include fragments, truncates and fusion proteins (e.g. comprising fused immunoglobulin, receptor, convertase or enzyme moieties).

The muteins of the invention also include proteins in which mutations have been introduced which effectively promote or impair one or more activities of the protein, for example mutations which promote or impair the function of a receptor, a recognition sequence or an effector binding site.

Muteins may be produced by any convenient method. Conveniently, site-directed mutagenesis with mutagenic oligonucleotides may be employed using a double stranded template (pBluescript KS II construct containing the RP-factor or RP-factor receptor/convertase gene), (e.g. Chameleon™ or QuikChange™ - Stratagene™). After verifying each mutant derivative by sequencing, the mutated gene is excised and inserted into a suitable vector so that the modified protein can be over-expressed and purified.

Preferred mutant forms are truncates consisting (or consisting essentially) of the RP-factor signalling domain or the RP-factor specificity-determining factor, or of the ligand binding domain of the RP-factor receptor, or combinations of two or more of the foregoing.

The invention also contemplates chimaeric RP-factors. These are factors which comprise one or more heterologous domains. In this context, a heterologous domain is a portion of an RP-factor which is derived from a different RP-

factor to that from which the other domain(s) with which it is associated are derived. Such chimaeric RP-factors find particular utility in applications where the specificity and/or activity of the RP-factor is manipulated or altered.

Useful in the construction of such chimaeric RP-factors are DNA fragments or cassettes consisting essentially of DNA encoding selected domains (for example, the signalling domain or the specificity-determining domain), the fragment or cassette optionally being bounded by one or more restriction endonuclease cleavage sites or cloning sites. The invention also contemplates concatenated domain cassettes, as well as mutant RP-factor structural genes which have cloning sites (e.g. one or more restriction endonuclease cleavage sites) located in one or more interdomain regions.

The term equivalent as used herein and applied to the materials of the invention defines materials (e.g. proteins, DNA etc.) which exhibit substantially the same functions as those of the materials of the invention while differing in structure (e.g. nucleotide or amino acid sequence). Such equivalents may be generated for example by identifying sequences of functional importance (e.g. by identifying conserved or canonical sequences or by mutagenesis followed by functional assay), selecting an amino acid sequence on that basis and then synthesising a peptide based on the selected amino acid sequence. Such synthesis can be achieved by any of many different methods known in the art, including solid phase peptide synthesis (to generate synthetic peptides) and the assembly (and subsequent cloning) of oligonucleotides.

The homologues, fragments, muteins, equivalents or derivatives of the proteins of the invention may also be defined *inter alia* as those proteins which cross-react with antibodies to the proteins of the invention, and in particular which cross-react with antibodies directed against any of the specific proteins shown Fig. 1.

The invention also contemplates all individual functional domains of the RP-factors of the invention as separate and independent entities.

The invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and or host cells described *infra*).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the RP-factor or RP-factor receptor/convertase (or homologue, species variant, allelic form, derivative, mutein or equivalent thereof) of the invention.

A pharmaceutical composition is a solid or liquid composition in a form, concentration and level of purity suitable for administration to a patient (e.g. a human or animal patient) upon which administration it can elicit the desired physiological changes. The vaccines of the invention may include any suitable adjuvant (e.g. Freund's adjuvant, BCG or BCG extracts).

In another aspect, the invention relates to a pharmaceutical composition comprising the material of the invention which is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

In another aspect, the invention relates to an antibody (or antibody derivative) specific for the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention.

The antibody is preferably in a form suitable for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or formulated in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration. The antibody may be labelled and/or immortalised and/or conjugated to another moiety, and such embodiments find particular utility in diagnostic applications.

According to another aspect of the invention there is provided an isolated or recombinant RP-factor receptor.

The receptor/convertase may be derived from any of the sources hereinbefore described, for example from a bacterial source (e.g. a pathogenic bacterial source). Such sources include high G+C Gram-positives, Micrococcus spp. (e.g. M. luteus); or Mycobacterium spp. (for example a fast- or slow-growing mycobacterium, e.g. M. tuberculosis, M. leprae, M. smegmatis or M. bovis); or Streptomyces spp. (e.g. S. rimosus); or Corynebacterium spp. (e.g. C. glutamicum).

The invention also contemplates homologues, derivatives, muteins or equivalents of the receptors/convertases of the invention, as well as recombinant RP-factor receptors/convertases (as hereinbefore defined).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention.

Preferably, the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) or pharmaceutical

composition is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an antibody (or antibody derivative) specific for the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention. The antibody may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an RP-factor antagonist or inhibitor.

Preferably, the antagonist or inhibitor comprises: (a) the antibody of the invention; and/or (b) the receptor/convertase of the invention; and/or (c) an RP-factor mutein comprising an RP-factor specificity-determining domain, which for example lacks a functional signalling domain.

The antagonist or inhibitor of the invention is preferably: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated by the invention is an RP-factor agonist, activator or mimetic. Preferably, the agonist, activator or mimetic comprises: (a) the RP-factor receptor/convertase antibody as herein described; and/or (b) an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; and/or (c) an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or (d) operably coupled combinations of any of (a)-(c).

The agonist, activator or mimetic may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) formulated in a pharmaceutical excipient, a unit dosage form, in a form suitable for local or systemic administration or in admixture with an antibiotic.

Preferably, the agonist, activator or mimetic may be for use in adjunctive therapy (for example formulated or presented in combination with an antimicrobial agent, e.g. an antibiotic).

The invention also contemplates isolated nucleic acid encoding the RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) or RP-factor receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) of the invention. The nucleic acids of the invention therefore embrace DNA having any sequence so long as it encodes the proteins of the invention. It will be appreciated by those skilled in the art that as a result of degeneracy in the genetic code, any particular amino acid sequence of the invention may be encoded by many different sequences. Thus, the nucleic acid sequence may be selected or optimised, e.g. with respect to the codon usage in any particular host cell.

The invention also contemplates vectors (e.g. an expression vectors) comprising the nucleic acid of the invention. The nature of the vector is not critical to the invention. Any suitable vector may be used, including plasmid, virus, bacteriophage, transposon, minichromosome, liposome or mechanical carrier.

The expression vectors of the invention are DNA constructs suitable for expressing DNA which encodes the desired protein product (e.g. RP-factor or RP-factor receptor) which may include: (a) a regulatory element (e.g. a promoter, operator, activator, repressor and/or enhancer), (b) a structural or coding sequence which is transcribed into mRNA and (c) appropriate transcription, translation, initiation and termination sequences.

Particularly preferred are vectors which comprise an expression element or elements operably linked to the DNA of the invention to provide for expression thereof at suitable levels. Any of a wide variety of expression elements may be used, and the expression element or elements may for example be selected from promoters, enhancers, ribosome binding sites, operators and activating sequences. Such expression elements may comprise an enhancer, and for example may be regulatable, for example being inducible (*via* the addition of an inducer).

As used herein, the term "operably linked" refers to a condition in which portions of a linear DNA sequence are capable of influencing the activity of other portions of the same linear DNA sequence. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The vector may further comprise a positive selectable marker and/or a negative selectable marker. The use of a positive selectable marker facilitates the selection and/or identification of cells containing the vector.

Also contemplated by the invention are host cells comprising the vector of the invention. Any suitable host cell may be used, including prokaryotic host cells (such as Escherichia coli, Streptomyces spp. and Bacillus subtilis) and eukaryotic host cells.

In another aspect, the invention provides a culture or transport medium comprising the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example Mycobacterium spp. Streptomyces spp. and Corynebacterium spp.

The invention also contemplates a nucleic acid probe comprising nucleic acid complementary to the nucleic acids of the invention. Such probes are preferably selectively hybridisable with nucleic acid encoding the proteins (e.g. the RP-factors of RP-factor receptors/convertases) of the invention. They are conveniently single stranded DNA or RNA probes.

The invention also contemplates a diagnostic kit comprising the factor (or homologue, derivative, mutein or equivalent thereof), receptor, antibody, probe or culture medium of the invention.

In another aspect, the invention contemplates antisense DNA corresponding to the nucleic acid encoding the RP-factor or RP-factor receptor/convertase of the invention.

The invention also contemplates a process for producing an antimicrobial drug comprising the steps of: (a) providing an RP-factor receptor; (b) providing candidate drugs; (c) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs and determining the affinity of the candidate drug for the RP-factor receptor, wherein the affinity is an index of antimicrobial activity, and optionally (d) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (c).

Preferably, the process for producing an antimicrobial drug comprises the steps of: (a) providing an RP-factor receptor/convertase; (b) providing a candidate drug; (c) providing an RP-factor; (d) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs in the presence of the RP-factor, and then determining the ability of the candidate drug to compete non-productively with the RP-factor for binding to the RP-factor receptor, wherein the competitive binding ability is an index of antimicrobial activity, and optionally (e) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (d).

The invention also covers an antimicrobial drug produced by (or obtainable by) the processes of the invention, and also derivatives thereof.

Also contemplated by the invention is a method for determining the microbiological quality of a product (e.g. a foodstuff, pharmaceutical preparation or medical product) comprising the step of contacting a sample of the product with an RP-factor (for example, an RP-factor as hereinbefore defined). In such methods, the RP-factor preferably forms part of a nutrient composition (e.g. a plate, broth, film or dipstick).

In another aspect, the invention relates to a method of culturing bacterial (e.g. mycobacterial) cells, comprising the step of incubating the cells in a culture medium containing an RP-factor (for example, an RP-factor as hereinbefore defined).

Also contemplated by the invention is an *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with an RP-factor (for example, an RP-factor as hereinbefore defined).

The diagnostic method of the invention preferably includes the step of incubating the culture or transport medium of the invention to permit growth of cells in the biological sample (e.g. bacterial cells).

Also contemplated by the invention is a method of: (a) stimulating the growth of a microorganism; and/or (b) resuscitating a dormant, moribund or latent microorganism; comprising the step of contacting the microorganism with an RP-factor (for example, an RP-factor as hereinbefore defined).

The invention also contemplates a process for producing the recombinant RP-factor or RP-factor receptor/convertase of the invention comprising the steps of: (a) culturing the host cell of the invention, and (b) purifying the factor or receptor/convertase from the cultured host cells (e.g. from a culture supernatant or cell fraction).

Also contemplated by the invention is a process for producing the recombinant RP-factor or receptor/convertase of the invention comprising the steps of: (a) probing a gene library with a nucleic acid probe which is selectively hybridisable

with the cognate structural gene to produce a signal which identifies a gene that selectively hybridises to the probe; (b) expressing the gene identified in step (a) (for example by cloning into a host cell, e.g. according to the process as hereinbefore defined) to produce the factor or receptor.

Also covered is a recombinant RP-factor or receptor/convertase obtainable by the above-described process.

Medical applications

The invention permits the isolation, synthesis and rational design of a wide range of novel medicaments and pharmaceuticals for use in therapy, prophylaxis and diagnosis.

The various forms of therapy, prophylaxis and diagnosis in which the materials of the invention find application may involve changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway of one or more infecting pathogens.

Thus, the materials of the invention find general application as antimicrobial agents, for example as antibacterial agents. They may therefore be used in the treatment, prophylaxis or diagnosis of microbial (e.g. bacterial) infections, particularly those infections associated with latency (e.g. mycobacterial infections).

Thus, the invention may for example be used to prevent, reduce or interfere with: (a) the resuscitation of a latent (or dormant) pathogen, and/or (b) the growth of a pathogen, and/or (c) the multiplication and spread of a pathogen; and/or (d) the activation of a latent infection (for example a latent bacterial (e.g. mycobacterial) infection).

In general, the materials of the invention may be used to treat conditions in which changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway or blockading the RP-factor receptor/convertase associated with an infecting pathogen is indicated.

Particularly useful materials for use in such therapies/prophylactic methods include RP-factor antagonists or inhibitors. Such antagonists or inhibitors may comprise antibodies to the RP-factor or to the RP-factor receptor/convertase as herein defined; the RP-factor receptor/convertase of the invention; an RP-factor mutein, e.g. which comprises an altered RP-factor specificity-determining domain and/or which lacks a functional signalling domain.

RP-factor antibodies act to sequester and ultimately eliminate endogenous RP-factors in a patient bearing a latent microbial infection.

RP-factor receptor antibodies bind non-productively to the receptors associated with the infecting pathogen. Antibodies to the convertase inactivate (e.g. by steric inhibition) the convertase activity and so prevent maturation of the RP-factor. The antibodies may therefore competitively inhibit the binding of endogenous RP-factor to the receptors/convertases associated with the infecting pathogen. Alternatively, they may bind with high affinity (and/or essentially irreversibly) to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. A similar activity is displayed by the RP-factor muteins having altered specificity and/or signalling activity.

In either case, the RP-factor-RP-receptor/convertase binding required for resuscitation of latent pathogens, growth of the pathogen and/or progression of the disease state is perturbed, reduced or abolished.

RP-factor receptors for use as therapeutics in such methods are uncoupled from the signal transduction pathway with which they are normally associated. Thus, they are preferably free (i.e. in soluble or dispersible) form and/or not membrane bound. In this way, effective circulating or systemic concentrations of the free RP-factor receptor can be established and maintained in a patient. In this form, the RP-factor receptors act as RP-factor sinks, and titrate out (and preferably ultimately eliminate) endogenous RP-factors in a patient bearing a latent microbial infection. The receptors therefore reduce or prevent activation of the (latent) pathogen and/or stimulation of pathogen growth, so slowing or halting the progression of the infection.

In another aspect, the invention may be used to resuscitate or assist in resuscitating (or activate or assist in activating) a latent (dormant) pathogenic microbe *in vivo* to thereby potentiate adjunctive antimicrobial therapy. The adjunctive antimicrobial therapies for use in such applications are those which depend for full efficacy on a non-latent or active (e.g. growing or replicating) target pathogen population (for example adjunctive therapies based on certain types of antibiotic). Thus, the materials of the invention may act synergistically with various antimicrobial compounds in antimicrobial therapy.

In a preferred embodiment, the invention is used to potentiate the antimicrobial therapy of tuberculosis, for example involving co-administration of one or more of isoniazid, rifampicin, pyrzinamide and/or ethambutol (or streptomycin).

Particularly useful materials for use in such therapies include for example the RP-factors of the invention, RP-factor agonists, activators and mimetics. Such agonists, activators or mimetics may comprise: the RP-factor receptor antibodies as hereinbefore described; the RP-factor convertase as hereinbefore defined; an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or operably coupled combinations thereof.

The RP-factor receptor antibodies for use in such methods are those which serve to trigger an efferent signal transduction pathway at the RP-factor receptor. They may therefore act as RP-factor mimetics, breaking latency/dormancy and acting to resuscitate the pathogen.

Particularly useful in such methods are mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified). Such mutant RP-factors may be active against a broad range of pathogens (e.g. against substantially all pathogenic or infective mycobacteria) or targeted against specific pathogens (for example, M. tuberculosis and M. leprae).

The antibodies, RP-factors, receptors and convertases discussed above may be administered directly or *via* a live vaccine vehicle. Such live vaccines vehicles comprise microorganisms which have been genetically engineered to express (and preferably secrete) the therapeutically active antibodies, RP-factors, receptors and convertases of the invention *in vivo*.

The invention therefore finds application in the treatment of a wide variety of microbial infections, and finds particular application in the treatment of latent microbial (e.g. bacterial) infections.

In preferred embodiments, the invention finds application in the treatment of actinomycete or mycobacterial infections, for example those involving M. tuberculosis, M. leprae, M. bovis and M. avium.

Other infections which may be treated according to the invention include those involving Corynebacterium spp. (including Corynebacterium diphtheriae), Tropheryma whippelii, Nocardia spp. (including Nocardia asteroides and Nocardia brasiliensis), Streptomyces spp. (including Streptomyces griseus, Streptomyces paraguayensis and Streptomyces somaliensis), Actinomadura spp., Nocardiopsis spp., Rhodococcus spp., Gordona spp., Tsukamurella spp. and Oerskovia spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria.

The invention may also be embodied in various vaccines or immunotherapeutic agents. Such vaccines or agents target one or more elements of the RP-factor-RP-mediated signal transduction pathway described herein (and in particular, the RP-factor or RP-factor receptors/convertases themselves). Thus, the RP-factors may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against endogenous RP-factor in the patient, so reducing, preventing activation of the pathogen and so slowing or halting the progression of the infection.

Alternatively (or in addition), the RP-factor receptors/convertases may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against receptors for pathogen-borne RP-factor in the patient. In this way, cellular and/or humoral immune responses may be stimulated against the pathogen(s) and/or activation of a latent pathogen (or its continued growth or multiplication) *via* the RP-factor signal transduction pathway may be reduced or prevented, so slowing or halting the progression of the infection.

The invention also finds application in the preparation of live vaccines: attenuated microbial strains can be constructed in which the gene(s) encoding (or regulating the expression or activity of) one or more RP-factors are mutated. Such attenuated vaccines may be based on mutant strains of actinomycetes, mycobacteria (for example M. tuberculosis, M. leprae, M. bovis (such as *M. bovis* BCG) and M. avium), Corynebacterium spp. (including Corynebacterium diphtheriae), Tropheryma whippelii, Nocardia spp. (including Nocardia asteroides and Nocardia brasiliensis), Streptomyces spp. (including Streptomyces griseus, Streptomyces paraguayensis and Streptomyces somaliensis), Actinomadura spp., Nocardiopsis spp., Rhodococcus spp., Gordona spp., Tsukamurella spp. and Oerskovia spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria.

Particularly useful in such attenuated vaccines are strains bearing mutated RP-factor-encoding genes. Such mutations may be frameshift, deletion, insertion and/or substitution mutations. In preferred embodiments the mutations are null mutations (e.g. non-reverting null mutations), and may prevent growth of the microbe (i.e. "attenuate" it). In other embodiments the mutations may result in the expression of mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified) and/or which lack a functional signalling domain. Such mutant RP-factors may bind with high affinity (and/or essentially irreversibly) and non-productively to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. The attenuated microbial strains of the invention may also bear mutations in other

genes (for example, in other genes essential to growth), and may also bear one or more genetic marker elements.

Biotechnological applications

It is widely recognised that the great majority (probably well in excess of 99%) of soil organisms have not yet been cultured. Hitherto uncultured organisms are also expected to exist in other sources. The present invention may be used to permit the recovery of such organisms by culture from any source. Thus, the invention provides a way of unlocking an immense reservoir of biodiversity that is known to exist, but is presently inaccessible.

Thus, the present invention provides an unprecedented resource from which libraries of potentially useful microorganisms and biomolecules can be generated. Such libraries can then be used in screening methods to search for medically or industrially useful products.

Thus, in another aspect the invention provides a process for producing a library of biomolecules comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; and (c) isolating microorganisms from the culture of step (b).

The process may further comprise the step of screening the isolated microorganisms for those which elaborate one or more biomolecules of interest (for example a metabolite, enzyme, antibiotic (e.g. antiviral, antibacterial or antifungal agent) or toxin).

Also contemplated is a biomolecule produced by (or obtainable by) the above process, or a derivative thereof.

In another aspect, the invention provides a process for producing a library of microorganisms (e.g. bacteria) comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived sample); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; (c) isolating microorganisms from the culture of step (b).

Also contemplated is a microbe produced by (or obtainable by) the above process, or a derivative (e.g. mutant) thereof.

Exemplification

The invention will now be described in more detail with reference to several Examples. These are for exemplary purposes only and are not intended to limit the invention in any way.

Explanation of the Figures

Figure 1: Part A. Alignment of the predicted amino acid sequence of the *M. luteus* RP-factor with similar genes found in *M. tuberculosis* and *M.leprae*. Proteins similar to the RP-factor are derived from *M. tuberculosis* (accession nos. U38939, nt 2406-2765, and Z81368, nt 33932-34396) and *M. leprae* (accession nos. L01095, nt 12292-12759, and L04666, nt 25446-24921). The DNA sequences of interest in accession Z81368 are also encompassed by accession AD000010. N-terminal residues corresponding to predicted Gram-positive signal sequences are underlined. The *M. leprae* L04666 sequence may also contain a short, 32 aa signal peptide. Part B. Alignment between the predicted amino acid sequence of the *M. luteus* RP-factor and p60 proteins from *Listeria* spp. Many of the residues that are conserved in the alignment between the C-terminal portion of the *M. luteus* RP-factor (residues 125-220) and the *L. monocytogenes* EGD p60 protein (residues 158-245), are also conserved in the p60 protein from six other *Listeria* spp.

Figure 2: The sequence of the RP-factor-encoding gene and its predicted product. The nucleotide sequence is in lower case with PCR primers in bold. The predicted protein sequence is in upper case bold (single letter code). Protein and peptide microsequence data used for oligonucleotide design are in upper case italics.

Figure 3: The elution profile of the resuscitation activity. Fractions eluted from the DEAE-sepharose column (see Materials and Methods) with 0.25 M KCl were applied to a Mono Q column which was developed with a 20ml linear gradient from 0.08 to 0.28 M KCl in 10 mM Tris-Cl buffer supplemented by 10% glycerol, pH 7.4. 10 ml of a diluted suspension of starved cells (CFU 3.10^6cells.ml^{-1}, total count $1.2.10^9$cells.ml^{-1}) were added to 200 ml of LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract containing of 2µl of each fraction in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods. **A**: absorbance at 280nm and magnitude of KCl concentration. **B**: resuscitation activity. **C**: SDS-PAGE profile of the fractions following

DEAE-cellulose and Mono Q chromatography. Lanes : 1, markers (94,000, 67,000, 43,000, 30,000, 20,100, 14,400); 2, fraction from DEAE-cellulose column; 3, purifed preparation (fraction number 8 from the Mono Q - column). D: Reduction of apparent lag phase of viable cells. 10 µl of a diluted suspension of viable, stationary phase cells (viable count 20 cells) was added to 200 µl of LMM supplemented with 0.5 % w/v L-lactate and containing 2 µl of each fraction (from a different experiment to that shown in parts A and B) in 5-10 replicates in the Bioscreen instrument. The apparent lag phase was estimated by extrapolating the exponential growth line to the abscissa.

Figure 4: Effect of purified RP-factor on *M. luteus*. **A.** Concentration dependence of RP-factor activity for resuscitation: resuscitation of dormant cells with different concentrations of RP-factor. 10 ul of a diluted suspension of starved cells (CFU 3.10^6 cells.ml^{-1}, total count 5.10^9 cells.ml^{-1}) was added to 200 µl of LMM supplemented with 0.5 % w/v L-lactate, 0.05% yeast extract and RP-factor in concentrations shown in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods. **B.** Growth of washed cells. Stationary phase cells of *M. luteus* grown in LMM were washed five times by suspension and centrifugation in LMM from which lactate had been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted and inoculated into a 20 ml flask containing LMM or LMM plus 31 pM RP-factor. The initial cell density was 250 viable cells per ml and incubation was at 30°C with intensive shaking. Growth was monitored by plating 0.1ml samples on plates containing broth E solidified with agar.

Figure 5: Detection of RP-factor-like genes in *Micrococcus luteus*, *Mycobacterium smegmatis* and *Streptomyces rimosus*.

Part A *M. luteus*		Part B *M. luteus*	Part C
Lane 1	λ*Bst*EII	λ*Pst*I	λ*Pst*I
Lane 2	*Cla*I	*Xho*I	*S. rimosus Xho*I
Lane 3	*Sal*I	*Stu*I	*S. rimosus Stu*I
Lane 4	*Sac*II	*Sma*I	*S. rimosus Sma*I
Lane 5	*Pst*I	*Pvu*II	*S. rimosus Pvu*II
Lane 6	*Nco*I	*Pst*I	*S. rimosus Pst*I
Lane 7	*Nhe*I	*Kpn*I	*S. rimosus Bam*HI
Lane 8	*Mlu*I	*Bam*HI	*M. smegmatis Xho*I
Lane 9	*Aat*II	λ*Pvu*II	*M. smegmatis Stu*I
Lane 10 λ*Pst*I			*M. smegmatis Sma*I
Lane 11			*M. smegmatis Pvu*II
Lane 12			*M. smegmatis Pst*I
Lane 13			*M. smegmatis Bam*HI
Lane 14			λ*Pvu*II

Figure 6 : Effect of *M. luteus* RP-factor on the growth of *Mycobacterium smegmatis* (**A**) and *Mycobacterium bovis* (**B**) in batch culture as observed turbidimetrically. *M. smegmatis* was grown in broth E, to which was added RP-factor at 31 pM. Cells were inoculated at a level of ca 200 per well, and growth was monitored in the Bioscreen instrument. *M. bovis* was grown in Sauton medium, as described in the Materials and Methods section, to which RP-factor (620 pMol/L) was either added or not. The inoculum was ca 1.10^{-5} cells.ml^{-1}, and the OD shown is the average of 10 separate determinations of 10 separate tubes.

Examples

Material And Methods

Organisms and media.

Micrococcus luteus NCIMB 13267 (previously described as "Fleming strain 2665") was grown aerobically at 30°C in shake flasks in lactate minimal medium (LMM) containing L-lactate described previously (Kaprelyants and Kell (1993) Appl. Env. Microbiol. 59: 3187-3196; Kaprelyants and Kell (1992) J. Appl. Bacteriol. 72: 410-422). When the culture had reached stationary phase agitation was continued at 30°C for up to 2 months. Cultures were then held aerobically at room temperature without agitation for period for up to a further 2-3 months. The apparent initial viability of these cultures was less than 10^{-3}.

Mycobacterium smegmatis ("fast" strain, All-Russia State Institute for Contol of Veterinary Preparations, Moscow) was grown in nutrient broth E, while *Mycobacterium bovis* (BCG), *Mycobacterium tuberculosis* H37RV and *Mycobacterium avium* were grown in Sauton medium.

M. luteus *Spent medium preparation.*

Supernatant was obtained after the centrifugation of late logarithmic phase *M. luteus* cultures (200-1000 ml) grown in lactate minimal medium or in the same medium in which lactate was replaced by succinate plus 0.01% yeast extract from which macromolecules had been removed by dialysis. The inoculum consisted of 2% of cells grown in rich medium (Broth E, LabM) and then washed in LMM lacking lactate. The supernatants were passed through a 0.22 μm filter (Whatman) before use.

M. luteus *Cell viability by plating.*

Plates consisting of 1.3% Nutrient Broth E (LabM) or lactate minimal medium were used. Cell dilutions were made in quadruplicate with centrifuged and autoclaved spent medium taken from the starved culture. Plates were incubated at 30°C for 3-5 d.

M. luteus *Cell viability by MPN.*

The MPN assay was performed in a Bioscreen C optical growth analyzer (Labsystems, Finland) using lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract as a resuscitation medium. Dilutions of starved cells were made as described. 10 μl of each dilution (5-10 replicates) were added to a well containing 200 μl of either lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract or the same medium with fraction tested (2-20 μl). Growth (optical density) was monitored using a 600 nm filter. Plates were incubated at 30°C with intensive continuous shaking. The overall measurement period was 120h, each well being measured hourly. The fractions obtained after chromatography were dialysed against elution buffer 2 (see below), diluted in resuscitation medium in various proportions (1:10, 1:100, 1:500, 1:1000, 1:5,000, 1:10,000) and filtered through 0.22 μm Gelman filters before testing. The calculation of the MPN was based on published Tables.

Total cell counts

Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber.

Chromatography

Pre-wetted DEAE cellulose was added to culture supernatant (1:10 v/v) and incubated at 4°C for 1h with slow stirring. The cellulose was loaded into a column, and washed with 5 volumes of buffer 1 consisting of 10mM Tris-Cl, 1mM EDTA, 1mM DTT, 10% (v/v) glycerol, pH 7.4 with 10mM KCl. The column was eluted stepwise with 2-3 bed volumes of 0.3M KCl in buffer 1. The fraction obtained was slowly diluted with buffer 1 on ice to give a final KCl concentration of 0.08M. Forty column volumes of this fraction was then loaded onto a DEAE-sepharose fast flow column (1 part of sepharose pre-equilibrated with buffer 1 containing 0.08M KCl). The column was washed with 5 bed volumes buffer 1 containing 0.08M KCl and eluted stepwise with 3 volumes of 0.25M KCl in buffer 1. The fraction obtained was again slowly diluted with buffer 1 on ice to a final KCl concentration of 0.08M, filtered through a 0.22 μm Gelman filter and loaded onto a Mono Q column (model HR5/5, pre-packed, Pharmacia) equilibrated with buffer 2 consisting of 10mM Tris-Cl, 10% glycerol, pH 7.4 containing 0.08M KCl. The Mono Q column was eluted by a linear gradient from 0.08 M to 0.28 M KCl in buffer 2 (the total volume of the elution was 20 ml). The flow rate and fraction size were 1 ml/min and 1ml/tube respectively. All manipulations except the Mono Q chromatography step were performed at 4°C. The fractions obtained were dialysed against 10 mM Tris-Cl containing 10% glycerol (dialysis is important for the retention of activity) and stored at 4°C for up to 5 days without loss of activity. For prolonged storage in a deep freeze, fractions were dialysed in the same way and glycerol added to a final concentration of 20-30% w/v. The protein content in purified preparations was estimated by tryptophan fluorescence using lysozyme as a standard.

Trypsin treatment:

Trypsin was added to the active, dialysed fraction obtained from the mono Q column and diluted by LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract (1:100) (the final concentration of trypsin was 50 μg/ml). The mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of trypsin inhibitor (100 μg/ml). In control experiments trypsin inhibitor was added to the mixture (100 μg/ml) prior to incubation.

PAGE electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli. Chromatographic fractions were dialysed against 10mM Tris HCl, pH 7.4 for 4-5 h, dried in a speed-vacuum apparatus (1.5h), dissolved in sample buffer (Sigma, S-3401), loaded onto 15% acrylamide gel and run at a constant voltage of 200V . The gel was stained with colloidal Coomassie G (Sigma).

Chemicals.

Nutrient Broth E, yeast extract and agar were obtained from Lab M, whilst L-lactate (Li salt), succinate, trypsin, soybean trypsin inhibitor and DEAE-Sepharose fast flow were obtained from Sigma. DEAE cellulose DE52 was obtained from Whatman, and Mono S and Mono Q from Pharmacia. Other chemicals were of analytical grade and were obtained from Sigma or BDH.

DNA manipulations.

Protein microsequence data from the N-terminus (**ATVDTWDRLAE**exSNGTxD) and an internal peptide (VG**GEGYPHQA**SK) obtained from the purified RPF were used to design two oligonucleotides, denoted A1 [GCSACSGTSGACACSTGGGACCGSCTSGCSGAG] and A2 [GCYTGRTGIGGRTAICCYTCICC], respectively. The 147 bp PCR product obtained from *M. luteus* DNA with these two primers was labelled with digoxygenin and used as a probe for Southern hybridisation experiments. *Sma*I-digested genomic DNA was size-fractionated by agarose gel electrophoresis and circa 1.4 kbp fragments were cloned in pMTL20 and established in *E. coli* strain DH5α. Two recombinant plasmids carrying the desired insert were detected by hybridisation, confirmed by PCR using oligonulceotides A1 and A2, and one of them was manually sequenced on both strands using the dideoxy chain termination method.

Purification of RP-factor

RP-factor purified from culture supernatants of cells grown in lactate minimal medium, according to the protocol described in Materials and Methods, revealed the presence of a significant amount of polymeric material eluted from all types of columns used, which inhibited both the resuscitation of dormant cells and the growth of viable cells of *M.luteus*. Moreover, elevated concentrations of this material could even cause the lysis of cells (not shown). This inhibitory material appears to be a polymer derived from lactate, as lactate-containing LMM stored for 10 hours at room temperature without cells and subjected to the same procedure of purification revealed inhibitory properties similar to those of this spent medium. To avoid this problem we replaced lactate in the growth medium with succinate, although for good growth it proved necessary to add a small amount (0.01 % w/v) of yeast extract dialysed to remove macromolecules.

Using succinate-grown cultures, the active fraction was purified by a combination of anion exchange media (see Material and Methods). The final activity was eluted at around 180 mM KCl from a linear KCl gradient (from 0.08 to 0.28M KCl) on a MonoQ column in 3 adjacent fractions (Fig. 3). It is worth mentioning that it proved important to dialyse the fractions before testing their activity because some fractions were inactive before dialysis. Active fractions did not change their resuscitation activity after dilution up to 400 times (v/v).

Interestingly, those fractions which were active in causing resuscitation could also increase the growth rate of viable cells.

The resuscitation-promoting material from the final purification step was checked by SDS-PAGE. The final product (Fig. 3C) proved to consist of a single protein with a molecular weight estimated to be ca 16kD. All active fractions consist of single band with maximum content of protein in fraction N9.

Cloning of the RP-factor gene

Two primers were designed from protein microsequence data obtained for the N-terminus of the purified RP-factor and for an internal peptide. They were used to amplify a 147 bp fragment of *M. luteus* DNA, which was cloned and sequenced. The complete gene was then obtained by a combination of inverse PCR using oligonucleotides G1 and G2 and isolation of a 1.4 kbp *Sma*I genomic restriction fragment. Sequencing revealed that the original PCR product was part of a gene capable of encoding a protein having a signal sequence (Fig. 2). The predicted size of the secreted form of the gene product is 19,148 Dal, and its predicted N-terminal amino acid sequence agrees with the protein microsequence data, including residues that were not used in primer design (Fig. 2). The fact that the predicted gene product is larger than the RP-factor purified from culture supernatants suggests that it may, for example, be secreted as a precursor which is converted to its biologically active form upon contact with its cognate receptor/convertase.

Identification of RP-factor homologues

A BLAST search was undertaken using the predicted amino acid sequence of the ORF from *M. luteus* as query. Four genes with substantial similarity have been sequenced previously. Two each are found in *M. tuberculosis* and *Mycobacterium leprae* (Fig. 1A). At least one gene product in each organism appears to have a secretory signal sequence (underlined in Fig. 1A). The functions of the predicted products of these mycobacterial genes

are unknown; they were found by genome sequencing projects. The BLAST search also revealed similarity between residues 126-220 of the RP-factor and a conserved segment of the (major extracellular) p60 proteins that have been implicated in adherence of *Listeria* spp. to 3T6 mouse fibroblasts suggesting, perhaps, a possible role for the RP-factor or a proteolytic product thereof in adhesion in *M. luteus* (Fig. 1B).

In common with *M tuberculosis* and *M. leprae*, *M. luteus* contains a second gene similar to that encoding the RP-factor. Southern hybridisation experiments, using DNA samples cleaved with a range of different restriction enzymes, and the cloned 147 bp fragment as probe (Figs. 5A & B), reveal two hybridising bands. The stronger hybridisation signal may arise from the gene encoding the secreted RP-factor. The other gene presumably corresponds to the mycobacterial genes identified above whose products may lack a secretory signal sequence.

Southern hybridisation experiments, using the 147 bp fragment as probe, as well as PCR experiments, using two oligonucleotides based on highly conserved amino acid motifs as primers, indicate that genes encoding proteins similar to the RP-factor are of widespread occurrence, at least throughout Gram-positive bacteria whose DNA has a high G+C content. Similar genes are detectable by either or both of these methods in all six *Streptomyces* species we have tested, including *Streptomyces rimosus* (Fig. 5C) as well as in other mycobacteria, including *Mycobacterium smegmatis* (four similar genes - Fig. 5C), *Mycobacterium bovis* (BCG) and *Corynebacterium glutamicum* (2 similar genes). No homologues are present in other organisms, whose genomes have been (entirely or almost completely) sequenced (*Mycoplasma genitalium, Helicobacter pylori, Staphylococcus aureus, Saccharomyces cerevisiae, Escherichia coli, Methanococcus jannaschii, Bacillus subtilis, Haemophilus influenzae, Streptococcus pneumoniae* etc.).

Domain structure

The sequence information shows that the RP-factor gene and at least three of its mycobacterial homologues share a secretory signal sequence and a particularly highly conserved, ca. 70-residue segment. The latter is a candidate for a signalling domain. Most of this segment is weakly hydrophilic (Kyte-Doolittle) and is predicted to form amphipathic α-helical (Garnier-Robson; Chou-Fasman) or β-sheet regions (Eisenberg). Overall, the segment has a low surface probability (Emini). The C-terminal section, by contrast, is much less highly conserved and might be considered a better candidate for determining specificity (i.e. be a specificity-determining domain). By analogy with other protein signalling systems (e.g. many pro-hormones in animals, and systemin in plants) it is possible that the proximate signalling molecule is a proteolytically cleaved product.

Two acidic residues, D7 & E13 (numbering according to the *M. luteus* secreted protein), within this segment are absolutely conserved. The KAEQIKRAE segment (residues 51-59) represents an island of particularly high surface probability. These elements may form part of functional domains within the RP-factor protein.

The conserved domain contains four conserved tryptophan residues (one of which is in a region of high surface probability DTWDR - residues 4-8). In the complex between human growth hormone and its first bound receptor, interactions involving two surface-located tryptophan residues in the receptor account for more than 75% of the binding free energy of the complex (Clackson and Wells, Science 267, 383-386, 1995). The two conserved cysteine residues may form a disulphide bridge.

RP-factor Activity

As well as resuscitating dormant cells, the purified RP-factor from *M. luteus* has been tested for growth-stimulatory activity against *M. luteus* and several other organisms. It strongly stimulates the growth *of M. luteus* and *M. smegmatis* and it appears to have weaker activity on *M. tuberculosis*, *M. bovis* (BCG) and *M. avium* (see Fig. 6). In all cases, there is a shortening of the apparent lag phase in batch culture (see Figs. 3D, 4B, 6B and Table 1). The factor is active in poor media and in poor media supplemented with yeast extract and it loses activity after boiling or treatment with trypsin.

When ca. 40 pM RP-factor was added to washed cells of *Mycobacterium smegmatis*, growth occurred after 20-24 hr, whereas the control lacking RP-factor showed no growth after 6 days. Experiments with slowly growing mycobacteria yielded similar results. Growth of *M. bovis* (BCG) was also strongly stimulated by 40 pM RP-factor: growth occurred after 14 days whereas the control lacking RP-factor showed no growth after 90 days. Finally, RP-factor also stimulated the growth of *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium kansasii* (see Table 1).

Table 1. Purified *M. luteus* RPF stimulates growth of mycobacteria

Organism	Bacterial growth[$]	
	RPF omitted	RPF added
Mycobacterium tuberculosis H37RV	1 (4)	30 ±10 (4)
Mycobacterium avium	0 (3)	350 ±100 (3)
Mycobacterium kansasii	1 (3)	120 ±50 (3)

[$]Growth was estimated microscopically (magnification times 600) after 14 days of incubation; ca. 50 µl of each culture was fixed, stained using Ziehl-Neelsen reagent and counted. Values in the body of the Table are average numbers of cells in a microscope field ± standard deviation with the number of determinations in parentheses. RP-factor (after elution from the Mono Q column and dialysis) was used at a concentration of circa 40 pMol/L; activity was lost after either trypsin treatment, heating (autoclaving) or filtration through a 12 kDal cutoff membrane.

Isolation and characterisation of the gene encoding the second homologue from *M. luteus*

A combination of inverse PCR using oligos G1 and G2 (see Fig. 2) as primers, and cloning of suitably sized genomic restriction fragments, can be employed to isolate the gene encoding the second homologue from *M. luteus*. The sequence of the gene can then be determined, taking care to eliminate any possible PCR errors by analysis of genomic clones and direct sequencing of PCR fragments obtained by combining the products of multiple, independent PCR reactions. Comparative sequence analyses of the proteins from *M. luteus*, *M. leprae* and *M. tuberculosis* can then be used to refine predictions concerning residues, sequence motifs and structural motifs which may be important for biological function.

Over-expression and purification of *M. luteus* and *M. tuberculosis* gene products in *E. coli*

PCR primers can be designed, incorporating suitable restriction sites such that sequences encoding the secreted forms of the *M. luteus* and the *M. tuberculosis* RP-factors can be amplified and inserted, in the correct reading frame, into commercially available plasmids (pET or pCAL vectors). The PCR-amplified fragments can first be cloned in a pBluescript KS II vector (Stratagene) so that their entire sequence can be verified, to eliminate possible PCR errors. (This material can also be employed for site-directed mutagenesis - see *infra*) The pET or pCAL constructs can then be employed to obtain controlled expression of large quantities of histidine- or calmodulin binding peptide-tagged proteins that can be purified, essentially to homogeneity, in a single step. Finally, the tags used in protein purification can be removed (using enterokinase or thrombin, as appropriate).

Antibody preparation

Purified RP-factor can be injected into rabbits and polyclonal antibodies produced. Alternatively, monoclonal antibodies can be produced using established techniques.

CLAIMS:

1. Isolated RP-factor.

2. The factor of claim 1 which is a secreted RP-factor.

3. The factor of claim 1 which is a non-secreted RP-factor (e.g. a cell-associated or cytosolic factor).

4. The factor of any one of the preceding claims which is derived from a bacterium (e.g. a pathogenic bacterium).

5. The factor of claim 4 which is derived from a high G + C Gram-positive bacterium.

6. The factor of claim 5 which is derived from:
(a) Micrococcus spp. (e.g. M. luteus); or
(b) Mycobacterium spp. (for example a fast- or slow-growing mycobacterium, e.g. M. tuberculosis, M. leprae, M. smegmatis or M. bovis); or
(c) Streptomyces spp. (e.g. S. rimosus); or
(d) Corynebacterium spp. (e.g. C. glutamicum).

7. A homologue, derivative, allelic form, species variant, mutein or equivalent of the factor of any one of the preceding claims.

8. A factor of any one of the preceding claims which comprises (or consists of) the RP-factor signalling domain.

9. A factor of any one of the preceding claims which comprises (or consists of) the RP-factor specificity-determining domain.

10. Recombinant RP-factor, wherein the RP-factor is for example as defined in any one of the preceding claims.

11. A pharmaceutical composition (e.g. a vaccine) comprising an RP-factor as an active ingredient (for example the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of the preceding claims), the RP-factor for example being present at a concentration sufficient to confer biological activity on the pharmaceutical composition.

12. An RP-factor (for example the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) or pharmaceutical composition as defined in any one of the preceding claims) which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

13. An antibody (or antibody derivative) specific for the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of the preceding claims.

14. The antibody of claim 13 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

15. Isolated RP-factor receptor or convertase.

16. The receptor/convertase of claim 15 which is derived from a source as defined in any one of claims 4-6.

17. A homologue, derivative, allelic form, species variant, mutein or equivalent of the receptor/convertase of claim 15 or 16.

18. Recombinant RP-factor receptor/convertase, wherein the receptor/convertase is for example as defined in any one of claims 15-17.

19. A pharmaceutical composition (e.g. a vaccine) comprising the receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of claims 15-18.

20. The receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent) or pharmaceutical composition as defined in any one of claims 15-19 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or

(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

21. An antibody (or antibody derivative) specific for the receptor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of claims 15-20.

22. The antibody of claim 21 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

23. An RP-factor antagonist or inhibitor.

24. The antagonist or inhibitor of claim 23 which comprises:
(a) the antibody of claim 13, 14, 21 or 22; and/or
(b) the receptor of claims 15-20; and/or
(c) an RP-factor mutein which comprises an altered RP-factor specificity-determining domain or which lacks a functional signalling domain.

25. The antagonist or inhibitor of claim 23 or 24 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

26. An RP-factor agonist, activator or mimetic.

27. The agonist, activator or mimetic of claim 26 which comprises:
(a) the antibody of claim 21 or 22; and/or
(b) an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; and/or
(c) an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or
(d) an RP-factor convertase; and/or
(e) operably coupled combinations of any of (a)-(d).

28. The agonist, activator or mimetic of claim 27 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form, in a form suitable for local or systemic administration or in admixture with an antibiotic.

29. The agonist, activator or mimetic of claim 28 which is for use in adjunctive therapy (for example in combination with an antibiotic).

30. Isolated nucleic acid encoding the RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) or RP-factor receptor as defined in the preceding claims.

31. A vector (e.g. an expression vector) comprising the nucleic acid of claim 30.

32. A host cell comprising the vector of claim 31.

33. A culture or transport medium comprising an RP-factor (e.g. the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in the preceding claims), for example comprising a culture supernatant containing an RP-factor.

34. A nucleic acid probe comprising nucleic acid complementary to the nucleic acid of claim 30.

35. A diagnostic kit comprising an RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent), receptor, antibody, probe, culture supernatant or culture medium as defined in any one of the preceding claims.

36. Antisense DNA corresponding to the nucleic acid of claim 30.

37. A process for producing an antimicrobial drug comprising the steps of:
(a) providing an RP-factor receptor;
(b) providing candidate drugs;
(c) screening the candidate drugs by contacting the RP-factor receptor with one of the candidate drugs and determining the affinity of the candidate drug for the RP-factor receptor, wherein the affinity is an index of antimicrobial activity, and optionally
(d) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (c).

38. A process for producing an antimicrobial drug comprising the steps of:
(a) providing an RP-factor receptor;
(b) providing a candidate drug;
(c) providing an RP-factor;
(d) screening the candidate drugs by contacting the RP-factor receptor with one of the candidate drugs in the presence of the RP-factor, and then determining the ability of the candidate drug to compete non-productively with the RP-factor for binding to the RP-factor receptor, wherein the competitive binding ability is an index of antimicrobial activity, and optionally
(e) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (d).

39. An antimicrobial drug produced by (or obtainable by) the process of claim 37 or 38, or a derivative thereof.

40. A method for determining the microbiological quality of a product (e.g. a foodstuff, pharmaceutical preparation or medical product) comprising the step of contacting a sample of the product with an RP-factor (for example, an RP-factor as defined in the preceding claims).

41. A method of culturing bacterial (e.g. mycobacterial) cells, comprising the step of incubating the cells in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims).

42. An *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with an RP-factor (for example, an RP-factor as defined in the preceding claims).

43. The method of claim 42 wherein the biological sample is incubated with a culture or transport medium as defined in claim 33.

44. A method of:
(a) stimulating the growth of a microorganism; and/or
(b) resuscitating a dormant, moribund or latent microorganism;
comprising the step of contacting the microorganism with an RP-factor (for example, an RP-factor as defined in the preceding claims).

45. A process for producing the RP-factor or RP-factor receptor of the invention comprising the steps of:
(a) culturing the host cell of claim 32, and
(b) purifying the factor or receptor from the cultured host cells (e.g. from a culture supernatant or cell fraction).

46. A process for producing the RP-factor or receptor of the invention comprising the steps of:
(a) probing a gene library with a nucleic acid probe which is selectively hybridizable with the nucleic acid of claim 30 to produce a signal which identifies a gene that selectively hybridises to the probe;
(b) expressing the gene identified in step (a) (for example by cloning into a host cell, e.g. according to a process as defined in claim 45) to produce the factor or receptor.

47. An RP-factor or receptor obtainable by the process of claim 45 or 46.

48. A process for producing a library of biomolecules comprising the steps of:
(a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived);
(b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture;
(c) isolating microorganisms from the culture of step (b).

49. The process of claim 48 further comprising the step of screening the isolated microorganisms for those which elaborate one or more biomolecules of interest (for example a metabolite, enzyme, antibiotic (e.g. antiviral, antibacterial or antifungal agent) or toxin).

50. A biomolecule produced by (or obtainable by) the process of claim 48 or 49, or a derivative thereof.

51. A process for producing a library of microorganisms (e.g. bacteria) comprising the steps of:
(a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived sample);
(b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture;
(c) isolating microorganisms from the culture of step (b); and optionally
(d) culturing and/or mutagenising the microorganism.

52. A microorganism produced by (or obtainable by) the process of claim 51, or a derivative (e.g. mutant) thereof.

53. Use of a culture supernatant (or fraction or extract thereof) containing an RP-factor for:
(a) diagnosis, prophylaxis or therapy; or
(b) producing a library of microorganisms (e.g. according to the method of claim 51); or
(c) producing a library of biomolecules (e.g. according to the method of claim 48); and/or
(d) resuscitating a dormant, moribund or latent pathogen (e.g. according to the method of claim 44(b)).

54. A culture supernatant (or fraction or extract thereof) containing an RP-factor for use in therapy, prophylaxis or diagnosis.

55. An *ex vivo* method of diagnosis comprising the step of incubating a sample with a culture supernatant (or fraction or extract thereof) containing an RP-factor (or an RP-factor as defined in any one of the preceding claims) at a concentration sufficient to promote the recovery of microrganisms from the sample by culture.

56. The method of claim 55 wherein the sample:
(i) is from an accessible body site, for example a mucous membrane of the vagina, anus, nose, urethra, cervix, skin, conjunctiva, mouth or throat; and/or
(ii) comprises a fluid or semi-solid (for example a bodily fluid or semi-solid, e.g. discharge, vomit, secretion, excreta, sputum or blood); and/or
(iii) comprises a solid (e.g. stool, tissue, food or biopsy sample); and/or
(iv) comprises a culture (e.g. a microbiological culture).

57. A live vaccine comprising an attenuated microbe, which microbe bears a mutation in a gene encoding (or regulating the expression or activity of) one or more RP-factors.

58. The vaccine of claim 57 wherein the microbe selected from any of: an actinomycete, mycobacterium (for example M. tuberculosis, M. leprae, M. bovis (e.g. *M. bovis* BCG) and M. avium), Corynebacterium spp. (e.g. Corynebacterium diphtheriae), Tropheryma whippelii, Nocardia spp. (e.g. Nocardia asteroides and Nocardia brasiliensis), Streptomyces spp. (e.g. Streptomyces griseus, Streptomyces paraguayensis and Streptomyces somaliensis), Actinomadura spp., Nocardiopsis spp., Rhodococcus spp., Gordona spp., Tsukamurella spp. and Oerskovia spp. and a pathogenic high G+C Gram-positive bacterium.

59. The vaccine of claim 57 or claim 58 wherein the mutation is selected from any of: frameshift, deletion, insertion and/or substitution mutations.

60. The vaccine of any one of claims 57-59 wherein the mutation:
(a) comprises a null mutation (e.g. a non-reverting null mutation); and/or
(b) prevents growth of the microbe; and/or
(c) results in the expression of a mutant RP-factor having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified) and/or which lacks a functional signalling domain.

SEQ 43

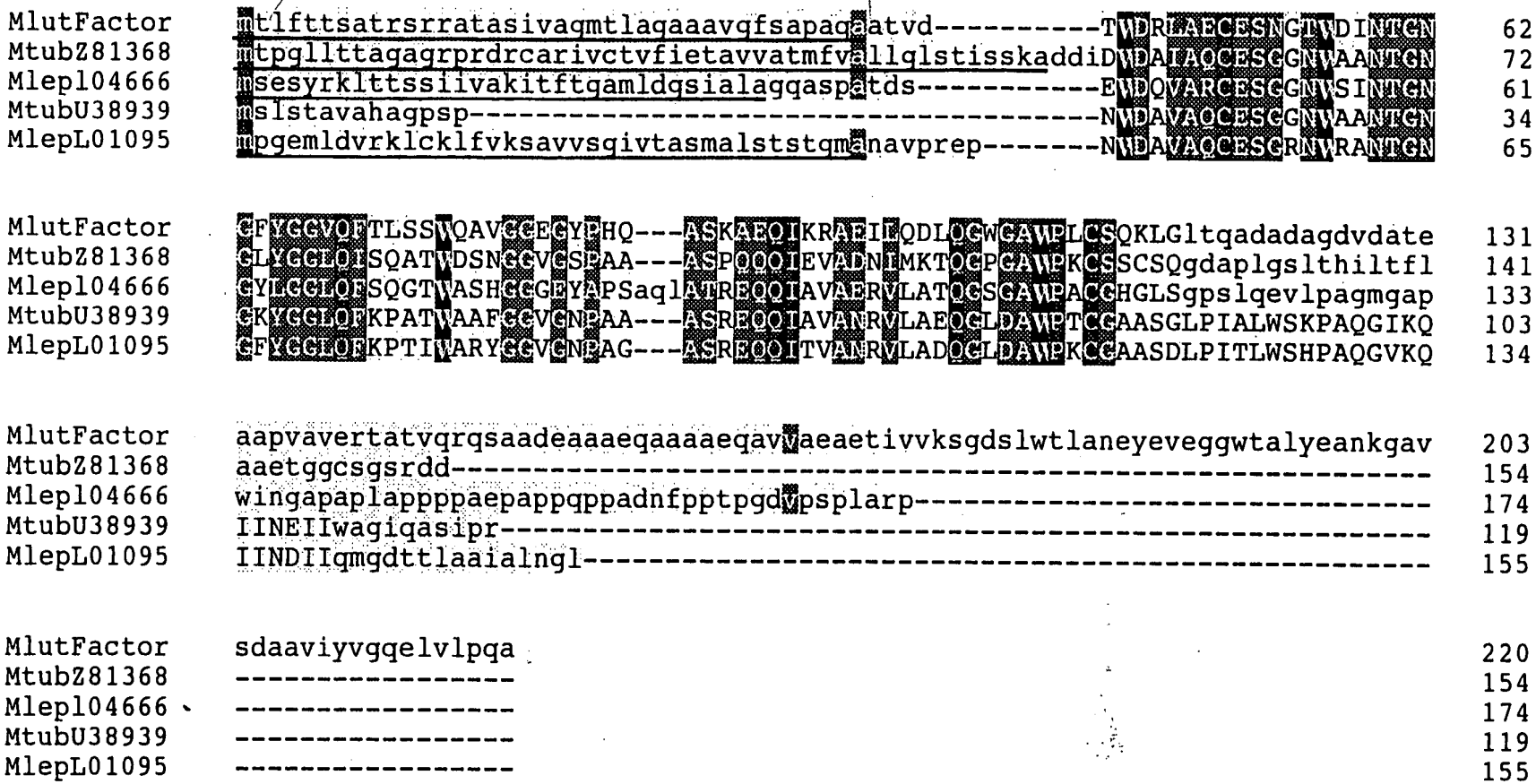

Fig. 14

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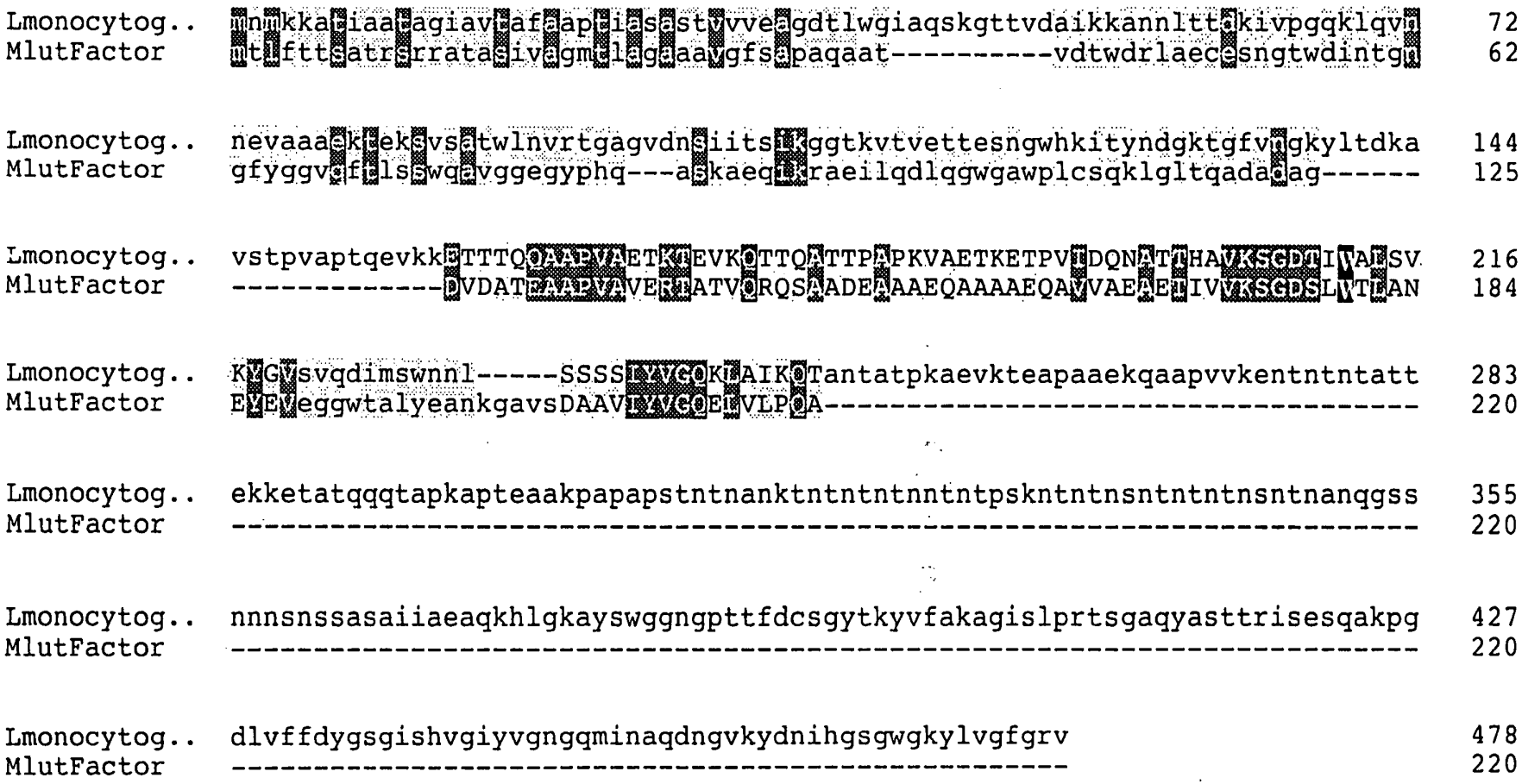

Lmonocytog.. mnmkkatiaatagiavtafaaptiasastvvveagdtlwgiaqskgttvdaikkannlttakivpgqklqvn 72
MlutFactor mtlfttsatrsrratasivagmtlagaaavgfsapaqaat----------vdtwdrlaecesngtwdintgn 62
Lmonocytog.. nevaaaekteksvsatwlnvrtgagvdnsiitsikggtkvtvettesngwhkityndgktgfvngkyltdka 144
MlutFactor gfyggvgftlsswqavggegyphq---askaeqtkraeilqdlqgwgawplcsqklgltqadaaag------ 125
Lmonocytog.. vstpvaptqevkkETTTQQAAPVAETKTEVKQTTQATTPAPKVAETKETPVIDQNATTHAVKSGDTIVALSV 216
MlutFactor ----------DVDATEAAPVAVERTATVQRQSAADEAAAEQAAAAEQAVVAEAETIVVKSGDSLWTLAN 184
Lmonocytog.. KYGVsvqdimswnnl-----SSSSIYVGQKLAIKQTantatpkaevkteapaaekqaapvvkentntntatt 283
MlutFactor EVEVeggwtalyeankgavsDAAVIYVGQELVLPQA---------- 220
Lmonocytog.. ekketatqqqtapkapteaakpapapstntnanktntntntnntntpskntntnsntntntnsntnanqgss 355
MlutFactor ---------- 220
Lmonocytog.. nnnsnssasaiiaeaqkhlgkayswggngpttfdcsgytkyvfakagislprtsgaqyasttrisesqakpg 427
MlutFactor ---------- 220
Lmonocytog.. dlvffdygsgishvgiyvgngqminaqdngvkydnihgsgwgkylvgfgrv 478
MlutFactor ---------- 220

Fig. 1B

2/8

Sequence of the *rpf* gene and its predicted product

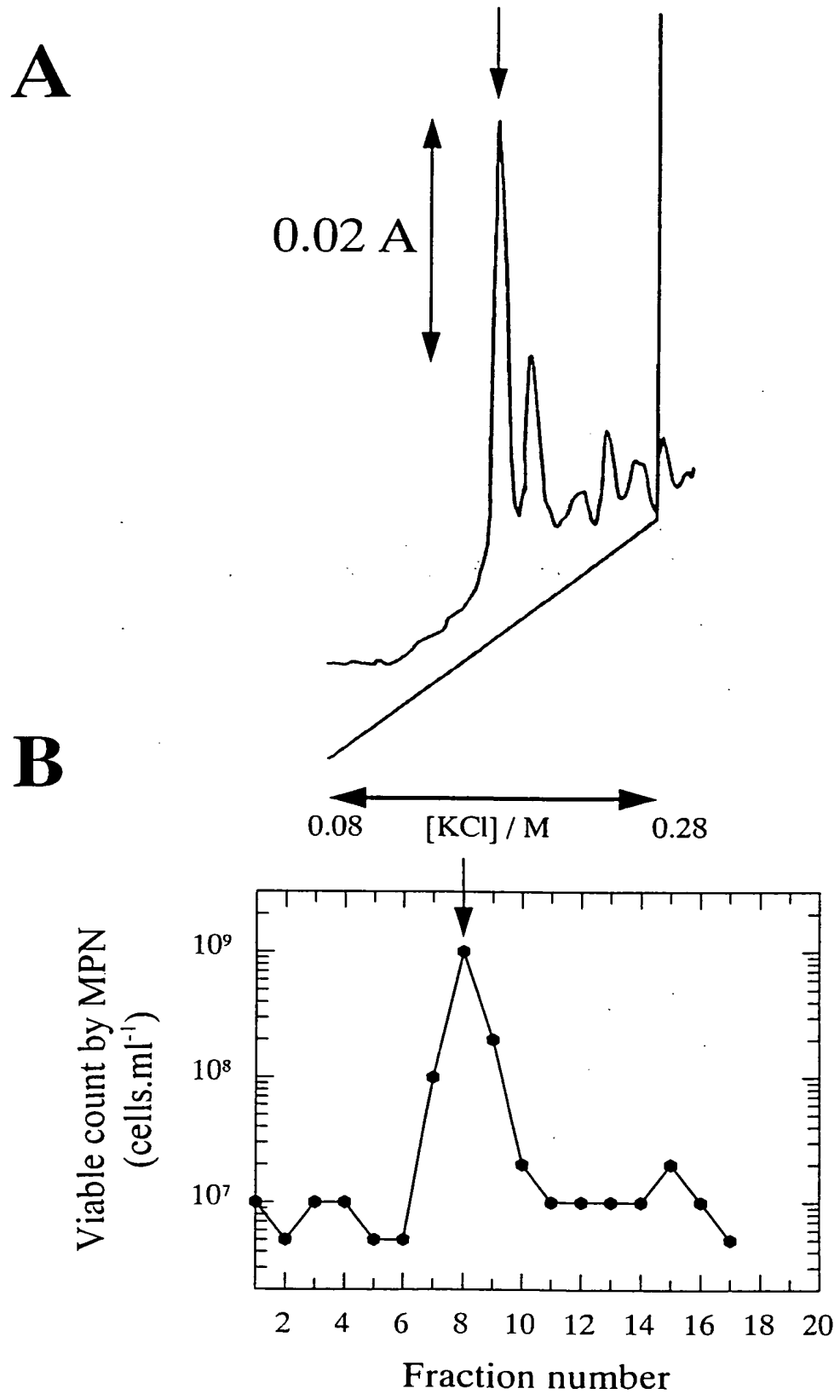
```
   1 accaaggagaaggacgaccccggtgtgcctcggccgccgatcagcgaggactcgccatgg 60
  61 acaccatgactctcttcaccacttccgccacccgctcccgccgtgccaccgcctcgatcg 120
        M  T  L  F  T  T  S  A  T  R  S  R  R  A  T  A  S  I  V
                                                              g
 121 tcgcgggcatgaccctcgccggcgccgccgccgtgggcttctccgccccggcccaggccg 180
       A  G  M  T  L  A  G  A  A  A  V  G  F  S  A  P  A  Q  A  A
                                                                 A
                              oligo A1>>>
     csacsgtsgacacstgggaccgsctsgcsgag
     ccaccgtggacacctgggaccgcctcgccgagtgcgagtccaacggcacctgggacatca 240
       T  V  D  T  W  D  R  L  A  E  C  E  S  N  G  T  W  D  I  N
       T  V  D  T  W  D  R  L  A  E  E  x  S  N  G  T  x  D
            <<< oligo G2                    oligo G1>>>
            gttgccgaagatgccgcc      agttcaccctgtcctcctg
 241 acaccggcaacggcttctacggcggcgtgcagttcaccctgtcctcctggcaggccgtcg 300
       T  G  N  G  F  Y  G  G  V  Q  F  T  L  S  S  W  Q  A  V  G
                                                              V  G
       <<< oligo A2
       ccictycciatrggigtrgtycg
 301 gcggcgaaggctacccgcaccaggcctcgaaggccgagcagatcaagcgcgccgagatcc 360
       G  E  G  Y  P  H  Q  A  S  K  A  E  Q  I  K  R  A  E  I  L
       G  E  G  Y  P  H  Q  A  S  K
 361 tccaggacctgcagggctggggcgcgtggccgctgtgctcgcagaagctgggcctgaccc 420
       Q  D  L  Q  G  W  G  A  W  P  L  C  S  Q  K  L  G  L  T  Q
 421 aggctgacgcggacgccggtgacgtggacgccaccgaggccgccccggtcgccgtggagc 480
       A  D  A  D  A  G  D  V  D  A  T  E  A  A  P  V  A  V  E  R
 481 gcacggccaccgtgcagcgccagtccgccgcggacgaggctgccgccgagcaggccgctg 540
       T  A  T  V  Q  R  Q  S  A  A  D  E  A  A  A  E  Q  A  A  A
 541 ccgcggagcaggccgtcgtcgccgaggccgagaccatcgtcgtcaagtccggtgactccc 600
       A  E  Q  A  V  V  A  E  A  E  T  I  V  V  K  S  G  D  S  L
 601 tctggacgctcgccaacgagtacgaggtggagggtggctggaccgccctctacgaggcca 660
       W  T  L  A  N  E  Y  E  V  E  G  G  W  T  A  L  Y  E  A  N
 661 acaagggcgccgtctccgacgccgccgtgatctacgtcggccaggagctcgtcctgccgc 720
       K  G  A  V  S  D  A  A  V  I  Y  V  G  Q  E  L  V  L  P  Q
 721 aggcctgagacgcctgaccggccccccggaccggtacc 758
       A  *
```

Key: The nucleotide sequence is in lower case with PCR primers in bold
The predicted protein sequence is in upper case with the secreted portion in bold and the signal sequence in normal type (single letter code)
Protein & peptide microsequence data used for oligonucleotide design are in upper case italics

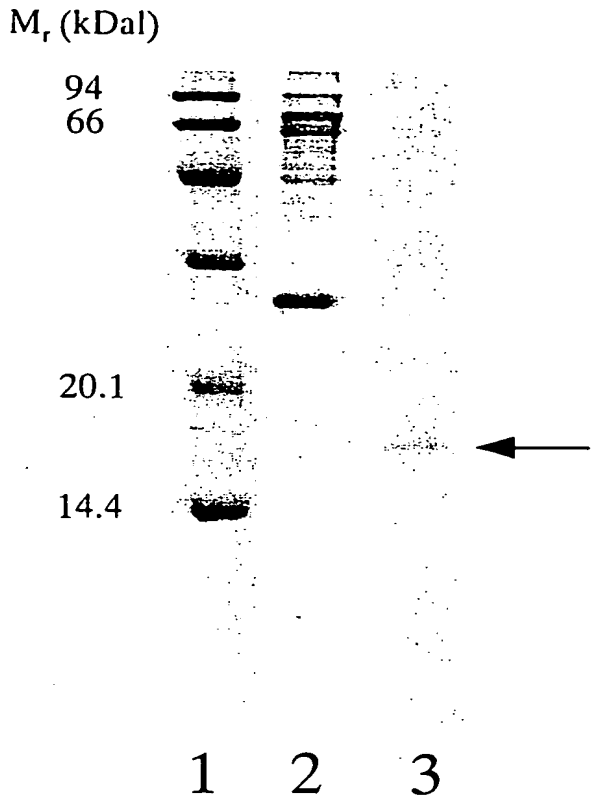
Predicted sequence of the secreted *rpf* gene product

```
1    ATVDTWDRLA ECESNGTWDI NTGNGFYGGV QFTLSSWQAV GGEGYPHQAS KAEQIKRAEI     60
61   LQDLQGWGAW PLCSQKLGLT QADADAGDVD ATEAAPVAVE RTATVQRQSA ADEAAAEQAA    120
121  AAEQAVVAEA ETIVVKSGDS LWTLANEYEV EGGWTALYEA NKGAVSDAAV IYVGQELVLP QA 182
```

Figure 3


A
0.02 A
B
0.08
[KCl] / M
0.28
Viable count by MPN
(cells.ml-1)
10⁹
10⁸
10⁷
2
4
6
8
10
12
14
16
18
20
Fraction number

C


M$_r$ (kDal)
94
66
20.1
14.4
1
2
3

D

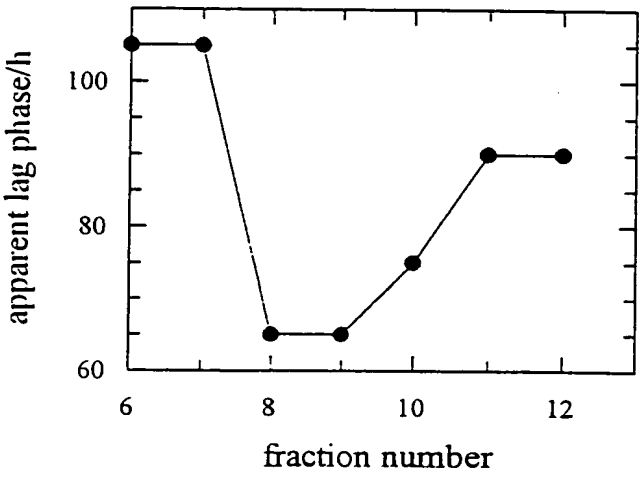
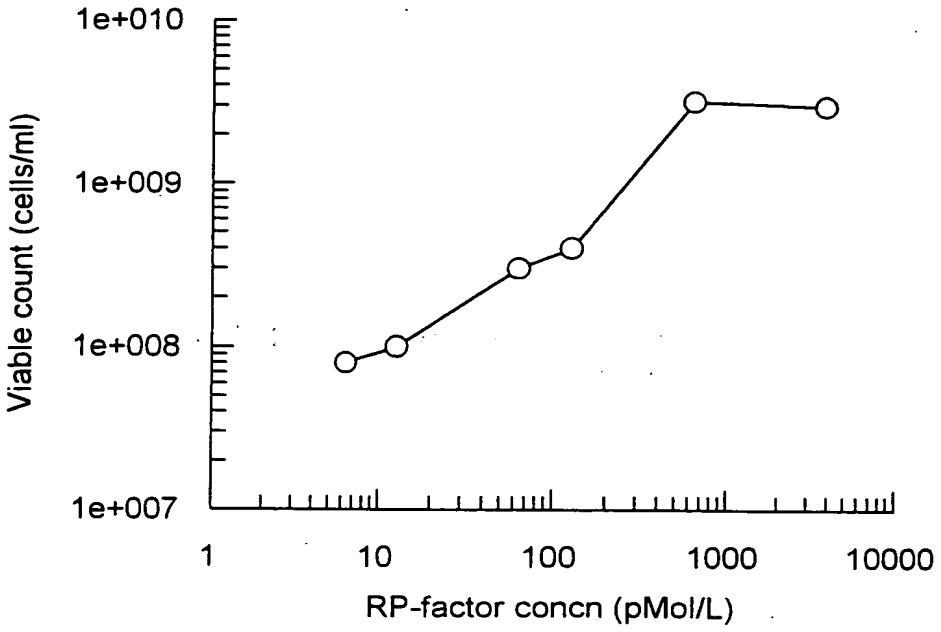

apparent lag phase/h
100
80
60
6
8
10
12
fraction number

FIGURE 4

A



The apparent viable count in the absence of RP-factor was 3.10^6 cells/ml.

B

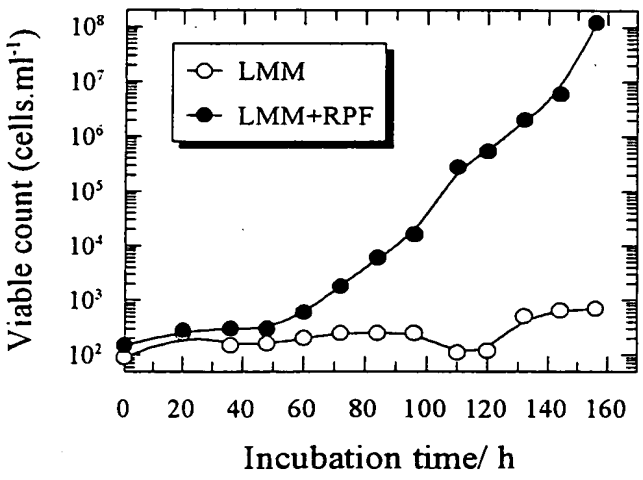


Figure 5

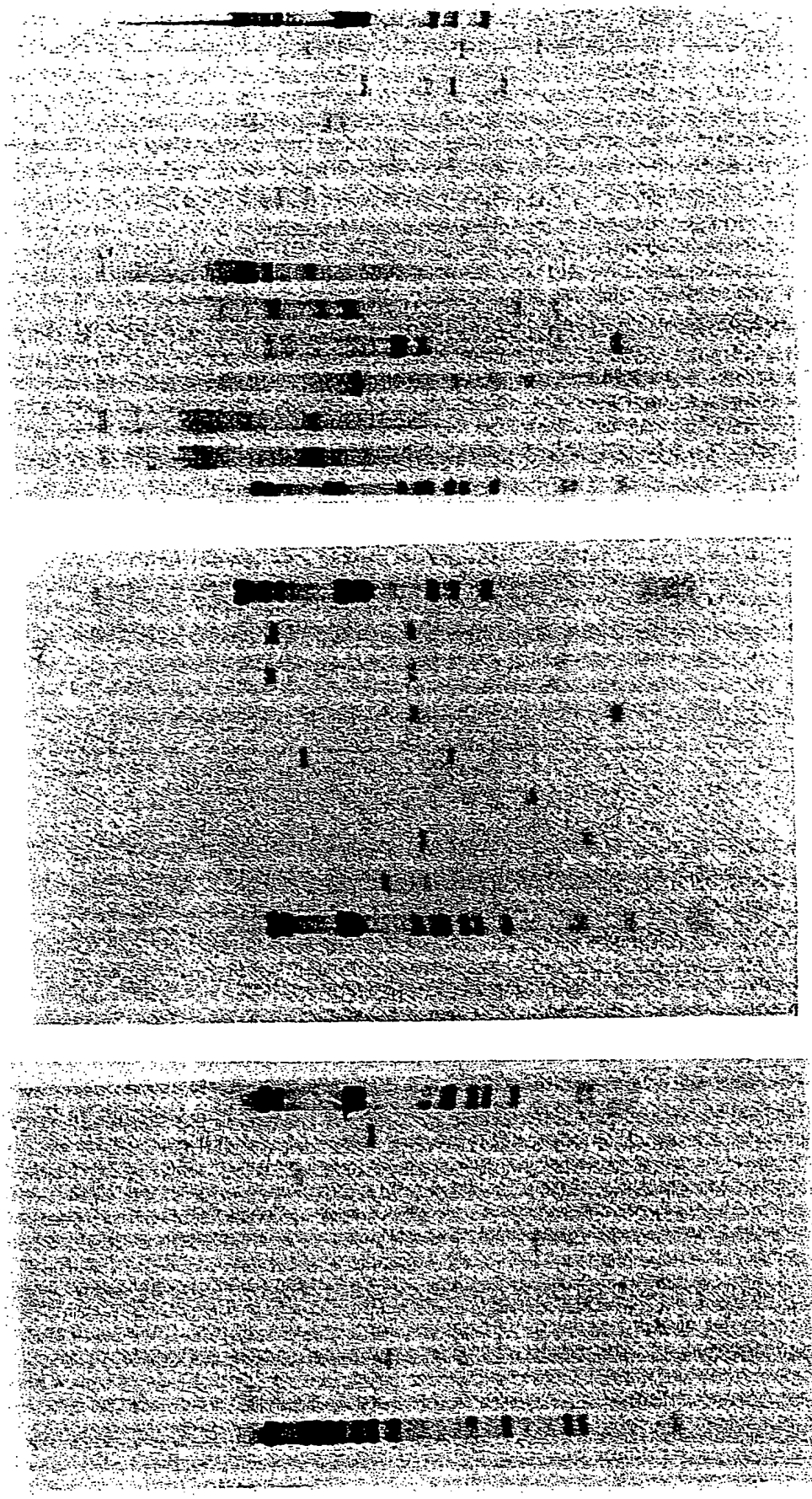
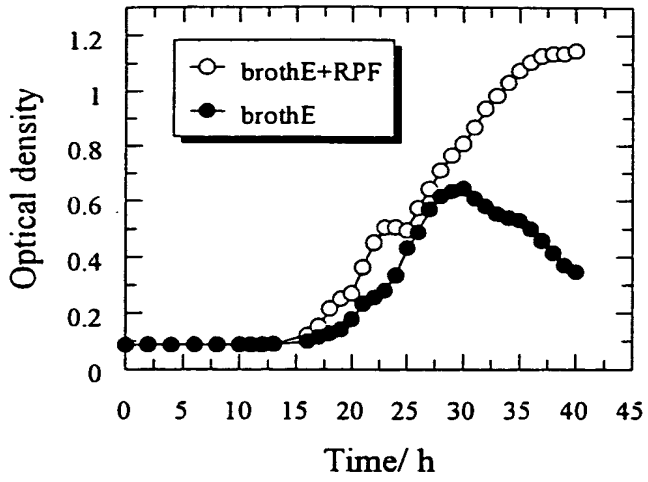
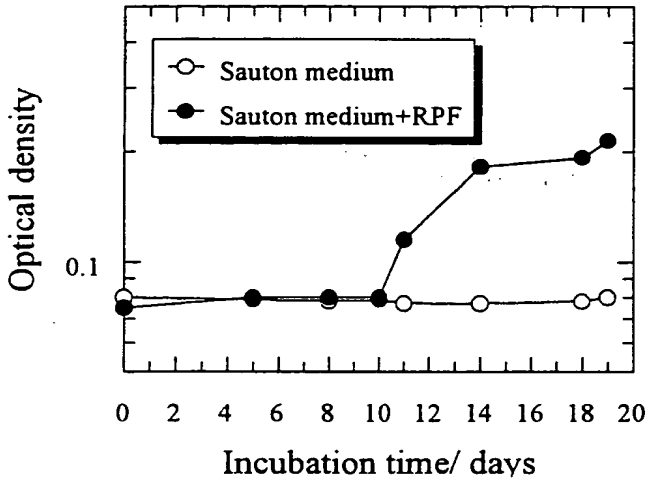


Figure 6

A


brothE+RPF
brothE
Optical density
1.2
1
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25 30 35 40 45
Time/ h

B


Sauton medium
Sauton medium+RPF
Optical density
0.1
0 2 4 6 8 10 12 14 16 18 20
Incubation time/ days

(1) 98/616,19

(2) 3-6-98

(3) [illegible]

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